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14. ABSTRACT

It is believed that certain breast tumors originate in either a breast stem or progenitorcell. Notably, tumors that arise in women carrying a BRCA1 gene mutation often exhibit a'basal' phenotype that may reflect their origin in the breast stem cell. We thereforehypothesized that the breast stem cell pool is aberrant in breast tissue of BRCA1 (or BRCA2)carriers versus non-carriers and that it becomes progressively and distinctively expanded inolder carriers. To evaluate this concept, we performed a pilot study in which we obtainedarchival samples from breast tissue from the Kathleen Cuningham Foundation Consortium forResearch into Familial Breast Cancer (kConFab). We first derived and analysed subpopulationsof breast tissue for the expression of putative stem cell markers and investigated means toderive short-term in vitro cultures. Our preliminary findings indicate that it is possible toidentify distinct subpopulations in normal breast tissue and tissue derived from BRCAmutations carriers. These studies our now being extended to study putative stem andprogenitor populations using reduction mammoplasties and prophylatic mastectomy specimens.

15. SUBJECT TERMS
Breast stem cells, BRCA1, Basal subtype, breast cancer

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INTRODUCTION

Preliminary work by our group and others *(1,2)* has led to the prospective isolation of mammary stem cells in mice. Interestingly, the mouse mammary stem cell-enriched population expresses basal markers and is negative for the steroid receptors ER and PR, as well as ErbB2 expression. This phenotype is reminiscent of the 'basal subtype' of breast cancer *(3)*. This observation lends support of the notion that basal tumors are derived from aberrant stem cells. The phenotype of the human breast stem cell has yet to be established. Nevertheless, certain markers have been identified that could enable the prospective fractionation of breast tissue (including ESA, MUC, CD44, CD24 and the capacity to efflux Hoechst dye), and it seems likely that a subset of cells selected using these or other markers will be enriched for breast stem cells.

Increasingly, certain breast tumors are believed to originate in either a breast stem or progenitor cell. Since tumors that arise in women carrying a BRCA1 gene mutation exhibit a 'basal' phenotype, we hypothesized that the breast stem cell pool is aberrant in breast tissue of BRCA1 (and possibly BRCA2) carriers versus non-carriers and that it becomes progressively and distinctively expanded in older carriers. The aims of our study were to evaluate stem cell characteristics in breast tissue derived from either BRCA1 or BRCA2 carriers, women with a strong family history where no mutation had been identified (nonBRCA1/2 women) and to compare these with breast tissue from normal women.

BODY

To evaluate the concept that the stem cell pool is perturbed in BRCA carriers, we performed a pilot study in which we obtained archival samples from breast tissue from the Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab). This consortium has accumulated over 1000 families with a strong family history of breast cancer and collects blood samples as well as tissue at the time of surgery (with patient consent), including prophylactic mastectomies. The proposed tasks for this project included:

1. Evaluation of putative breast epithelial stem cell markers
2. Derivation of short-term cultures
3. Establishment of tumor xenograft models using NOD/SCID mice

We first derived and analysed subpopulations of breast tissue for the expression of putative stem cell markers and investigated means to derive short-term *in vitro* cultures. Our preliminary findings indicate that it is indeed possible to identify distinct subpopulations in normal breast tissue and tissue derived from BRCA1 and BRCA2 mutation carriers and a representative example is shown in Figure 1. Cell suspensions were stained with a variety of cell surface markers. Dead cells were excluded on the basis of propidium iodide staining (PI) and further evaluation made on the PI low fraction (PI box in Figure 1). Next, haematopoietic and endothelial cells were excluded on the basis of CD45 and CD31 expression and lineage negative cells selected (third plot in Figure 1). Preliminary findings suggest that it is possible to fractionate the Lineage minus population on the basis of several markers. Two representative Markers, A and B are shown in the bottom plot in Figure 1.

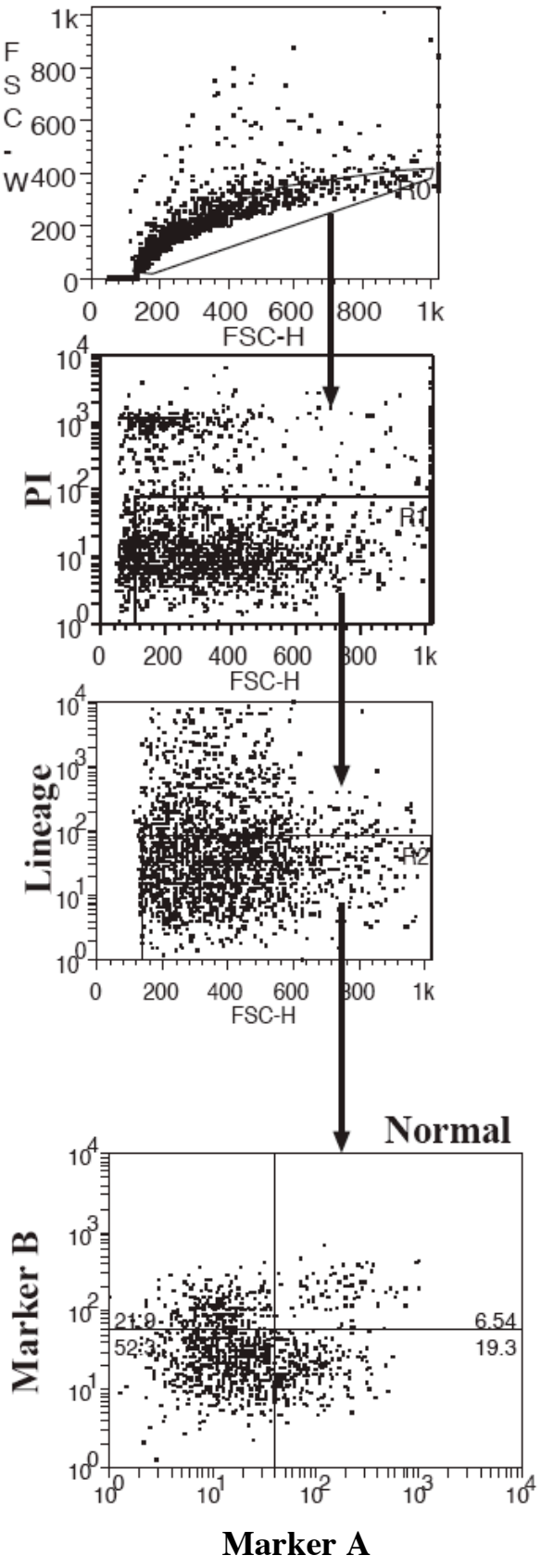

Figure 1. Fractionation of human breast tissue by Flow Cytometric Analysis.

These findings made during the period of DOD Concept grant support our now being extended to study putative stem and progenitor populations using reduction

mammoplasties and prophylatic mastectomy specimens, as we have since found that freshly prepared tissue is superior to samples that have been frozen in DMSO.

Short-term cultures have also been generated using archival material and a modified *in vitro* assay as previously described using mouse mammary epithelial cells *(1)*. Our study has revealed that different subpopulations generate colonies with variable efficiency, suggesting that some may be enriched for stem/progenitor capacity. (Figure 2).

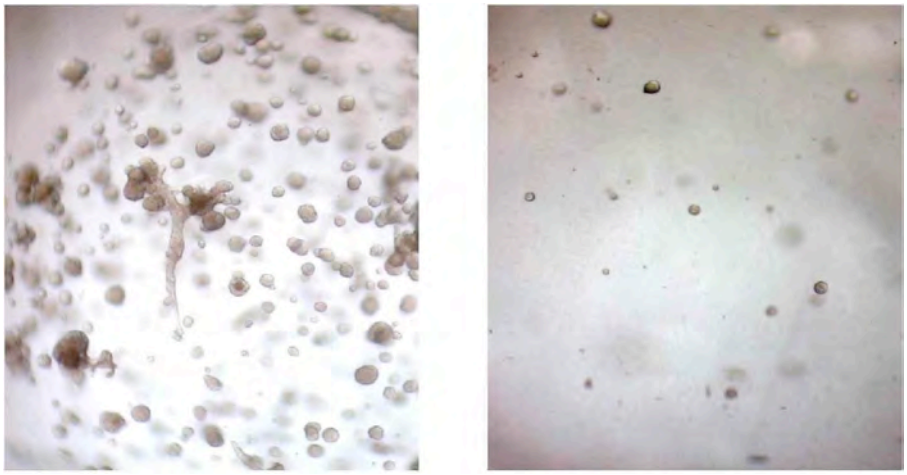

Figure 2. Derivation of short-term *in vitro* cultures. Subpopulations of cells were cultured in Matrigel using modified protocol as descfribed by Shackleton et al *(1)*. In the pilot experiment shown, one supopulation gave rise to numerous heterogeneous colonies (left panel). The right panel is a representative image of a subpopulation that gave rise to fewer colonies.

Insufficient sample numbers of suitable quality were available to determine whether there are qualitative differences between BRCA1, BRCA2, nonBRCA1/2 and control samples. However, this work will now be extended in future studies using prospectively collected tissue samples.

During the course of the DOD Grant funded period, we were able to establish the humanized NOD/SCID xenograft model in our laboratory. As 'proof of principle' mammary outgrowths were successfully derived using unsorted cell suspensions. One such outgrowth is shown in Figure 3. This work has placed us in a good position to prospectively collect fresh breast tissue and transplant sorted samples into recipient mice.

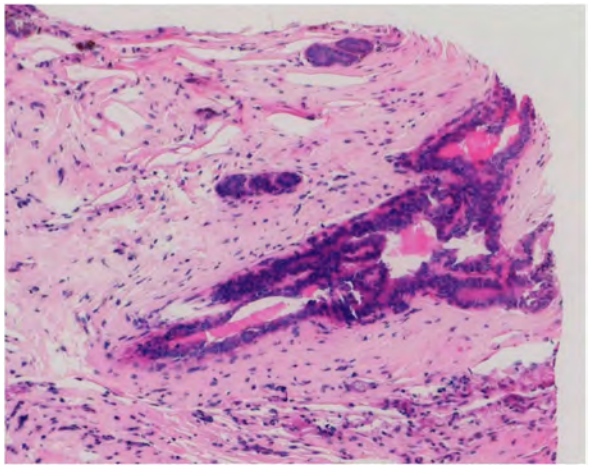

Figure 3. Histological section of a humanised mouse mammary fat pad. The fat pad, which was cleared of epithelial elements at puberty, contains an outgrowth that has arise from human epithelial cells.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a short-term *in vitro* culture system to assay subpopulations of breast tissue
- Establishment of the humanized NOD/SCID xenograft model to grow human breast tissue
- Identification of cell surface markers that define breast epithelial subpopulations

REPORTABLE OUTCOMES

Publications:

Pending – the concept award served as a 'pilot' project to establish model systems for the study of breast epithelial populations using archival material. We are now in a position to proceed with a more comprehensive project aimed at prospectively defining breast epithelial subpopulations using fresh breast tissue and to determine whether these are altered in women who harbor BRCA1 and BRCA2 mutations. This is necessarily a long-term project and the US DOD grant support will be recognised in future publication(s).

Abstracts and presentations:

Geoffrey Lindeman:

- 28th Annual Meeting, ANZ Breast Cancer Trials Group, Cairns, Queensland, Australia, July 2006 (Speaker)
  Title: How can we Improve the Breast Cancer Treatment Paradigm?
  Author(s): Lindeman GJ
  Abstract: none

- Familial Cancer – Research and Practice 2006, Annual Scientific Meeting, Couran Cover, Queensland, August 2006 (Speaker)
  Title: Negative hormone receptor status of mouse mammary stem cells reminiscent of human basal breast tumours
  Author(s): Asselin-Labat M-L, Shackleton M, Stingl J, Vaillant F, Forrest NC, Eaves CJ, Visvader JE, Lindeman GJ
  Abstract: refer appendix

Jane Visvader:

- Australian Stem Cell Centre Symposium, Melbourne, September 2006 (Speaker)
  Title: Breast stem cells and mammopoiesis
  Author(s): Visvader JE
  Abstract: none

- 3rd PacRim Breast and Prostate Cancer Meeting, Fraser Island, Queensland, Australia, October 2006 (Speaker)
  Title: Stem cells in mammopoiesis and breast cancer
  Author(s): Visvader JE
  Abstract: none

François Vaillant:

- Familial Cancer – Research and Practice 2005, Annual Scientific Meeting, Couran Cover, Queensland, August 2006 (Speaker)
  Title: Generation of a functional mammary gland from a single cell
  Author(s): François Vaillant, Mark Shackleton, Kaylene J. Simpson, John Stingl, Gordon K. Smyth, Marie-Liesse Asselin-Labat, Li Wu, Geoffrey J. Lindeman, Jane E. Visvader
  Abstract: refer appendix

Degrees supported in part by this award:
Dr Elgene Lim – Postgraduate Scholar (consumables component)

Development of animal models:
Development of xenograft model to assist growth of human breast tissue *in vivo*.

Funding applied for based in part on work supported by this award:
National Breast Cancer Foundation, Concept Award (A$150, 000)

CONCLUSION:

The work carried out to date has revealed that we will be able to prospectively identify subpopulations of epithelial cells using tissue derived from human breast (and tumor) tissue. Preliminary work, initiated with the support of the US DOD, has also shown that it will be possible to investigate these subpopulations using a range of markers, *in vitro* culture assays and xenograft models. Future studies will allow us to determine whether the subpopulations are indeed modified in the preneoplastic phase of cancer as well as determining whether breast tumors represent an expansion or modification of a specific subpopulation. This should form a basis for addressing the fundamental question of which cell type is the 'cell of origin' that gives rise to distinct forms of breast cancer, and could potentially provide a means to address the possibility that breast cancers contain a subset of 'tumor propagating cells' (cancer stem cells) *(4-6)*. The elucidation of novel markers of cancer stem cells could potentially serve as novel therapeutic targets in breast cancer.

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(4) Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414(6859):105-111.

(5) Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100(7):3983-8.

(6) Visvader JE, Lindeman GJ. Mammary stem cells and mammopoiesis. Cancer Res 2006;66(20):9798-801.

APPENDIX – meeting abstracts

Negative hormone receptor status of mouse mammary stem cells reminiscent of human basal breast tumours

Asselin-Labat M-L[1], Shackleton M[1], Stingl J[2], Vaillant F[1], Forrest NC[1], Eaves CJ[2], Visvader JE[1], Lindeman GJ[1,3]
[1]*The Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia*
[2]*Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada*
[3]*The Royal Melbourne Hospital, Melbourne, Australia*

The identification of at least five subtypes of breast cancer on the basis of gene profiling studies has generated considerable interest in identifying the cell types from which these phenotypically distinct tumour types arise. It is possible that normal progenitors, already restricted in their differentiative potential, become transformed, or that stem cells may be initially targeted with additional transforming events occurring in later progenitors. These possibilities prompted us to investigate the expression of ERα, PR, ErbB2/Her2, and EGFR/ErbB1 in normal mouse mammary stem cells.

Using the specific cell surface markers CD24 and α6β1-integrin, we have identified a distinct subpopulation of mammary cells that is enriched for mammary stem cells and expresses basal markers, and a subpopulation comprising luminal cells and stem cell-derived colony-forming cell progeny. The expression of key signalling molecules in these two subpopulations was examined. We found that the basal population, which is enriched in mouse mammary stem cells, did not express ERα, PR, or ErbB2/Her2 but did express epidermal growth factor receptor EGFR/ErbB1, whereas the subset of cells enriched for luminal cells expressed ERα (37% of cells) and PR (40% of cells) but not ErbB2/Her2 or EGFR/ErbB1. Ovariectomy confirmed the importance of estrogen signalling to luminal cell proliferation but had no effect on the size of the mammary stem cell-enriched population.

Thus, mouse mammary stem cells are 'triple negative' for ERα, PR, and ErbB2 and express basal markers. The phenotype of the mammary stem cell-enriched population recapitulates several characteristics of the poor-prognosis basal subtype of breast cancer, which is commonly observed in *BRCA1* tumours. One possibility is that the stem cell, or an early progenitor, is the 'cell of origin' that gives rise to the basal subtype of breast cancer.

Generation of a functional mammary gland from a single stem cell

François Vaillant[1,2,*], Mark Shackleton[1,2,*], Kaylene J. Simpson[1,2], John Stingl[3], Gordon K. Smyth[1], Marie-Liesse Asselin-Labat[1,2], Li Wu[1], Geoffrey J. Lindeman[1,2,*], Jane E. Visvader[1,2,*]

*[1]The Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia; [2]Bone Marrow Research Laboratories, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia; [3]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, Canada. *Equal contribution.*

The mammary gland can be functionally regenerated in mice by transplantation of epithelial fragments/cells, providing evidence for the existence of self-renewing, multipotential mammary stem cells. Using specific cell surface markers and flow cytometry, we have identified a distinct subpopulation that is enriched for mammary stem cells, demonstrated by transplantation into cleared fat pads at limiting dilution. Remarkably, a single mammary epithelial cell from this population, carrying the *lacZ* transgene, was found to generate a complete mammary gland *in vivo*. These cells contributed to both the luminal and myoepithelial lineages in transplanted virgin mammary glands, and extensive lobuloalveolar units were generated during pregnancy. Serial transplantation of the clonal outgrowths also yielded complete mammary glands. Together, these data establish that single cells from the enriched population have multipotential and self-renewing capacity, properties that are a hallmark of stem cells.

It will be of interest to determine whether the stem cell is a target of transformation in different mammary tumorigenesis models and whether analogous populations exist in human breast tissue and in breast cancer. Since BRCA1 *tumours* often exhibit a 'basal phenotype', which has been attributed to have stem cell properties, we intend studying whether the stem cell compartment is expanded or perturbed in preneoplastic breast tissue and tumours from BRCA1/2 carriers.